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The Proteasome: Paradigm of a Self-Compartmentalizing Protease

Review

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Controlling Proteolysis through Compartmentalization

Protein degradation is a necessity for many reasons: Homeostasis must be maintained while cellular structures are continually rebuilt, in particular during development or in response to external stimuli. Proteins misfolded as a consequence of mutations or ensuing from heat or oxidative stress must be scavenged because they are prone to aggregation. Beyond these more mundane "housekeeping" functions, protein degradation provides a means to terminate the lifespan of many regulatory proteins at distinct times; amongst them are cyclins, transcription factors, and components of signal transduction pathways (for reviews, see Coux et al., 1996; Hilt and Wolf, 1996; Varshavsky, 1997). Moreover, the immune system relies on the availability of immunocompetent peptides generated by the degradation of foreign antigens (for reviews, see Goldberg et al., 1995; Heemels and Ploegh, 1995).

However, since protein degradation is also a hazard, it must be subject to spatial and temporal control in order to prevent the destruction of proteins not destined for degradation. A basic stratagem in controlling protein degradation is compartmentalization, that is, the confinement of the proteolytic action to sites that can only be accessed by proteins displaying some sort of degradation signal. Such a compartment can be an organelle delimited by a membrane, as in the case of the lysosome. Proteins to be degraded must be imported into the lysosome via specific pathways, and the hydrolases carrying out the task must also be sorted from other proteins and are translocated by means of transport vesicles.

Prokaryotic cells, possessing neither membrane-bound compartments nor vesicular transport systems, have developed a different form of compartmentalization, namely self- or autocompartmentalization (Lupas et al., 1997b). This principle is seen at work in several unrelated proteases that have all converged toward a common architecture in which proteolytic subunits self-assemble to form barrel-shaped complexes. These enclose inner cavities, which are several nanometers in diameter and harbor the active sites. Access to these inner compartments is usually restricted to unfolded polypeptides, which can pass through the narrow pores or channels guarding the entrance. The target proteins thus require interaction with a machinery capable of binding and presenting them in an unfolded form to the proteolytic core complexes; these interactions may be of either a transient or a continuous nature. Since protein folding and unfolding are closely related mechanistically, it is assumed, but not proven, that this task is performed by ATPase complexes, which bear some resemblance to the chaperonins and have been referred to as "reverse chaperones" or "unfoldases" (Lupas et al., 1993). Since their action requires the hydrolysis of ATP, protein degradation becomes energy-dependent, although the hydrolysis of the polypeptide chain itself is an exergonic process.

Self-compartmentalizing proteases are common in all three domains of life: archaea, bacteria, and eukarya. This bears testimony to an old evolutionary principle. In fact, contrary to organelles such as the lysosome, self-compartmentalizing molecular devices offer far greater flexibility: when equipped with the appropriate localization signals, they can be deployed to different cellular locations in the cytosol or in the nucleus, wherever their action is needed. The advances made in recent years in understanding the structure of the proteasome and its mechanism of action has helped to shape the concept of self-compartmentalization, and the proteasome became the paradigm of this form of regulation.

The 20S Proteasome: Core of the Proteolytic Machinery

20S Proteasomes Are Found in All Three Domains of Life

The first description of a "cylinder-shaped" complex with proteasome-like features dates back to the late sixties. The plethora of names given to it subsequently is a reflection of the problems that were encountered over a period of two decades in trying to define its biochemical properties and cellular functions. Enzymological studies revealed an array of distinct proteolytic activities and led to a consensus name, "multicatalytic proteinase" (Dahlmann et al., 1988). This name, however, was soon replaced by a new one, the proteasome (Arrigo et al., 1988), emphasizing its character as a molecular machine (for a brief account of the early history of this field, see Baumeister et al., 1997).

At about the same time, it was found that the occurrence of proteasomes was not restricted to eukaryotic cells. A compositionally simpler, but structurally strikingly similar proteolytic complex was found in the archaeon *Thermoplasma acidophilum* (Dahlmann et al., 1989), which later took a pivotal role in elucidating the structure and enzymatic mechanism of the proteasome. Meanwhile, proteasomes have been identified in several archaea (e.g., see Maupin-Furlow and Ferry, 1995), and the forthcoming sequence data of more genomes will reveal how common they are within this domain.

The first evidence for the existence of proteasomes in bacteria was provided by a database search and sequence comparison (Lupas et al., 1994), which indicated that two types of proteasomes might exist in bacteria, one represented by the *Escherichia coli hslV* gene (Chuang et al., 1993) and one by a gene (*prcB*) found in the *Mycobacterium leprae* genome. Subsequent biochemical and structural studies confirmed the existence

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of two types of proteasomes in bacteria: HslV and its homologs, although clearly related to (β-type) proteasomal subunits, form a simpler complex in which two six-membered rings form the proteolytic core and associate directly with an ATPase of the Clp family (ClpX/HslU) (Bochtler et al., 1997; Rohrwild et al., 1997). The second type, represented by *Mycobacterium* and *Rhodococcus*, is possibly restricted in its occurrence to actinomycetales. These proteasomes form a complex indistinguishable in its general architecture from eukaryotic or archaeal 20S proteasomes (Tamura et al., 1995; Zühl et al., 1997a). It is conceivable that the actinomycetales have acquired the proteasome genes by horizontal gene transfer after their separation from other Gram-positive bacteria, possibly from a eukaryotic organism, especially considering that many actinomycetales live in symbiotic association with eukaryotes or are pathogens (Lupas et al., 1997a). Under normal growth conditions, proteasomes or proteasome-related complexes appear not to be essential in eubacteria. *M. smegmatis* cells in which proteasome genes are deleted are viable and phenotypically indistinguishable from wild-type cells (Knipfer and Shrader, 1997). This is different from the situation in yeast, where the disruption of 13 out of the 14 proteasome genes is lethal (e.g., see Hilt and Wolf, 1995), but is consistent with the observation that proteasome-related genes were not found in some recently sequenced eubacterial genomes (e.g., *Mycoplasma genitalium*).

The 20S Core Structure Is Conserved from Archaebacteria to Eukaryotes

Archaeal proteasomes are built of 14 copies each of two different but related subunits, α and β, whereas eukaryotic proteasomes contain two copies each of 14 different subunits, which, based on their sequence similarity, can be divided into an α-type and a β-type group. The subunits are arranged into four seven-membered rings, with the α-type subunits forming the two outer rings, and the β-type subunits the two inner rings. Collectively, they form a barrel-shaped complex, 15 nm in length and 11 nm in diameter (Figure 1a), which encloses three internal cavities, approximately 5 nm in diameter bounded by four narrow constrictions. The central cavity is formed by the two adjacent β rings, while the two outer cavities (the "antechambers") are formed jointly by one α and one β ring.

As anticipated from their sequence similarity, the noncatalytic α- and the catalytic β-type subunits have the same fold (Löwe et al., 1995; Groll et al., 1997): a four-layer α and β structure with a central five-stranded β sandwich flanked on either side by α helices (Figure 1a). Helices 1 and 2 mediate the interaction of the α- and β rings (β-*trans*-α) by intercalating in a wedge-like fashion. Helices 3 and 4, which are located on the opposite side, provide the dominant contacts between the two β rings (β-*trans*-β). While this general architecture is the same in the *Thermoplasma* and in the yeast proteasome, there is a large number of additional specific α-*cis*, β-*cis*, β-*trans*-α, and β-*trans*-β contacts in the yeast proteasome as detailed in Groll et al., 1997. Together with the propeptides, these contacts seem to ensure that the assembly proceeds in an orderly fashion, that is, that each of the 14 different subunits takes its correct place (see below).

The main difference between α- and β-type subunits is due to a highly conserved N-terminal extension of the α-type subunits, part of which (residues 20–30) forms an α helix (H0) across the top of the central β sandwich (see Figure 1a). The function of the N-terminal extension is not clear, but its location at the top of the α rings close to the entrance to the antechambers indicates that it may be important for the translocation of substrate or for interactions between the proteasome and its regulatory complexes. Instead of this N-terminal extension, β-type subunits have prosequences of varying lengths, which are removed during proteasome assembly, thus rendering the cleft between the central β sandwich freely accessible from the central cavity. Although the proteasome fold was initially believed unique, it has subsequently been shown to be prototypical of a new family of proteins referred to as the Ntn (N-terminal nucleophile) hydrolases (Brannigan et al., 1995).

The Proteasome: A Threonine Protease

A common feature of the Ntn-hydrolases is a "single residue" catalytic center, which is freed by the autocatalytic removal of the prosequence. The identification of the N-terminal threonine of the β subunit of the *Thermoplasma* proteasome as both the catalytic nucleophile and the primary proton acceptor came as a surprise (Löwe et al., 1995; Seemüller et al., 1995), since, until then, the proteasome was widely assumed to be an unusual type of serine protease. Beside the N-terminal threonine, β subunits require several other residues (Glu-17, Lys-33, Asp-166) for activity, although their exact roles remain to be clarified. Lys-33 and Glu-17 form a salt bridge across the bottom of the active site and may participate in the delocalization of the threonine side-chain proton by forming a charge relay system. Further evidence supporting the role of the N-terminal threonine came from work with the antibiotic lactacystin, or more accurately, its active form clasto-lactacystin β-lactone (Dick et al., 1996a), which covalently reacts with the Thr-1 of a specific subset of β-type subunits of mammalian proteasomes (Fenteany et al., 1995). The lactacystin experiments as well as recent mutational studies with yeast and mammalian β-type subunits (Chen and Hochstrasser, 1996; Schmidtke et al., 1996; Arendt and Hochstrasser, 1997) confirmed a prediction made on the basis of sequence comparisons that the proteolytic mechanism is the same in eukaryotic and in *Thermoplasma* proteasomes (Seemüller et al., 1995). From the conservation pattern of the active site residues, it was further deduced that, of the seven β-type subunits in an individual eukaryotic proteasome, only three are proteolytically active (see Figure 2a). Since each of them is present in two copies, the number of active sites in the central cavity is 6, instead of 14 as in the *Thermoplasma* proteasome. The reasons for this reduction in the number of active sites are unclear, and also, the function of the "inactive" β-type subunits awaits further clarification. Mutagenesis and chemical modification (e.g., see Heinemeyer et al., 1993; Enenkel et al., 1994; Arendt and Hochstrasser, 1997) have shown

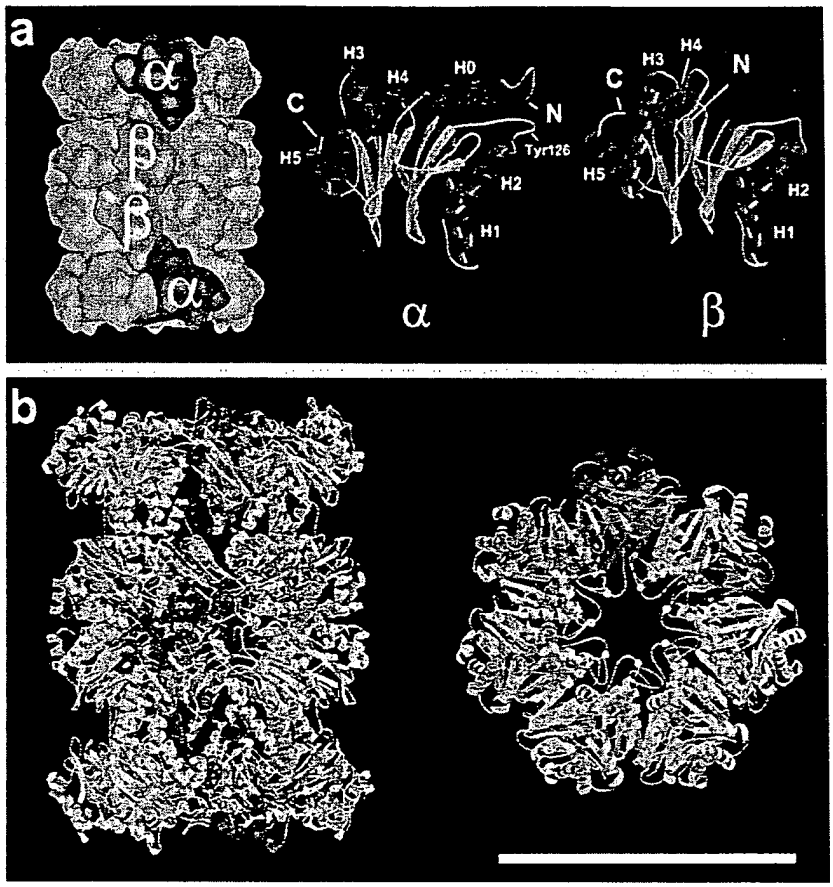


Figure 1. Structure of the *Thermoplasma* 20S Proteasome

(a) Low resolution model (~1.2 nm) derived from the atomic coordinates of the *Thermoplasma* proteasome (Löwe et al., 1995). The α subunits form the heptameric outer rings; the β subunits, the inner rings. This general architecture is conserved from *Thermoplasma* to higher eukaryotes (left). Individual α and β subunits are shown as ribbon drawings (right).

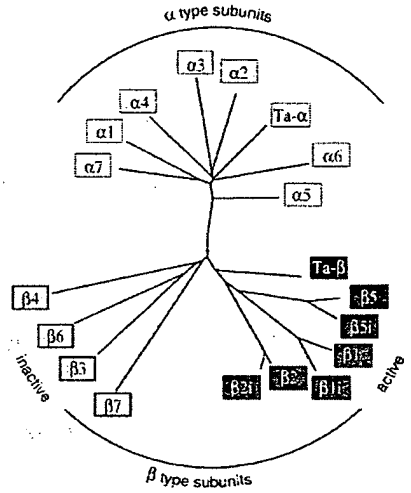
(b) Ribbon drawing of the 20S proteasome indicating one subunit in each of the four rings by different color coding (left). A half-proteasome (one α and one β ring) viewed down the 7-fold axis. The Tyr-126 residues in the loops between helix 2 (H2) and β-sheet 5 (S5), which line the polypeptide channel giving access to the interior, are marked by small white spheres (right). The scale bar is 10 nm.

that each of the three major activities of eukaryotic proteasomes (chymotrypsin-like activity, trypsin-like activity, and peptidylglutamyl peptide hydrolyzing activity), as defined by the degradation of short fluorgenic peptides, can be abolished by modifying either an active or an inactive subunit. It is not clear whether special pairs of active and inactive subunits exist, which interact allosterically, or whether mutations of inactive subunits simply tend to cause structural perturbations with a high likelihood of affecting the neighboring active subunits (see Figure 2b).

Access to the Central Cavity Is Restricted to Unfolded Proteins

Another feature apparently distinguishing the yeast proteasome and the *Thermoplasma* proteasome structure relates to the openings that give access to the inner cavities. The crystal structure of the *Thermoplasma* proteasome revealed the existence of a channel located along the 7-fold axis (Figure 1b). This channel is defined by a turn-forming segment with Tyr-126 at a strategic position; collectively, the seven turns form a hydrophobic annulus well suited for the translocation of an unfolded polypeptide. Direct evidence that this channel is the port of entry was obtained by electron microscopic visualization of a Nanogold-labeled insulin β chain caught in transit; the relatively bulky (approximately 2 nm) gold label prevents it from slipping across the channel into the antechamber (Wenzel and Baumeister, 1995). In the yeast proteasome, this channel is occluded by the N-terminal residues of the α subunits, which are disordered in the *Thermoplasma* proteasome. On the other hand, the yeast proteasome has small openings situated at the interface between α and β rings; this led to speculations that the side windows may be used to take up substrate. However, it is not very likely that such highly conserved structures use radically different routes for

a



c

new systematic name	traditional names yeast	human
α1	C7/PRS2	iota
α2	PRS4/Y7	C3
α3	PRS5/Y13	C9
α4	PRE6	C6/XAPC-7
α5	PUP2/DOA5	zeta
α6	PRE5	C2
α7	C1/PRS1	C8
β1	PRE3	Y/delta
β1i	-	LMP2/RING12
β2	PUP1	Z/alpha
β2i	-	MECL-1
β3	PUP3	C10
β4	PRE1/C11	C7
β5	PRE2/DOA3	X/MB1/epsilon
β5i	-	LMP7/RING10
β6	PRS3	C5
β7	PRE4	N3/beta

b

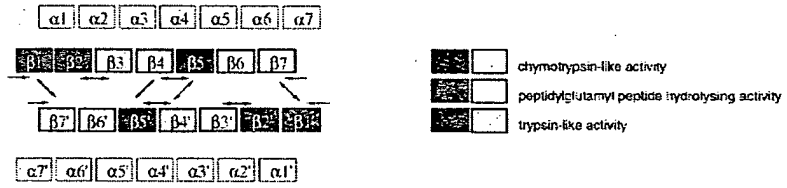


Figure 2. Relatedness and Topology of Subunits of Eukaryotic Proteasomes

(a) The dendrogram shows the classification of subunits into α and β types, and the division of β subunits into an "active" (closed boxes) and an "inactive" (open boxes) branch (for details, see Lupas et al., 1994). The terms active and inactive refer to the presence or absence of Thr-1 as the N-terminal nucleophile. For nomenclature, see (c). Ta-α and Ta-β represent *Thermoplasma acidophilum* α and β subunits, respectively.
(b) Localization of the 28 subunits within the eukaryotic proteasome. The diagram represents an unrolled cylinder of the 20S proteasome showing the positions of α and β subunits as determined by the crystal structure of the yeast proteasome (Groll et al., 1997). A mapping of the subunits of the human proteasome based on immunoelectron microscopy revealed a remarkably similar topology (Kopp et al., 1997). The color coding denotes pairs of active (closed boxes) and inactive (open boxes) subunits responsible for specific activities as deduced from mutagenesis experiments. Subunits forming such pairs are in direct contact to each other, either in *cis* (i.e., within one ring) or in *trans* (i.e., between two rings) as indicated by arrows. The prime symbol distinguishes equivalent subunits within the two α and the two β rings.
(c) Nomenclature of 20S proteasome subunits. The new systematic nomenclature is based on the location of the yeast subunits within the seven-membered rings relative to the C2 symmetry axis. The exchangeable subunits, which are present only in the immunoproteasomes of higher vertebrates, are marked with "i".

translocation. Moreover, the side windows would be rather ill-positioned for the uptake of polypeptides when the 20S proteasome associates with its regulatory complexes. It is more likely, therefore, that eukaryotic proteasomes use the central channel, which then must undergo a conformational change. It is noteworthy in this context that eukaryotic but not *Thermoplasma* proteasomes are purified in a latent state and need to be activated by chaotropic agents or heat. These treatments may selectively unfold the N-terminal sequence, resulting in an opening of the channel. Under physiological conditions, a gating of the channel could be controlled by regulatory complexes that dock to the two termini of the 20S complex. While the side windows are unlikely to be used for the entry of substrate, it is possible that they have a role in discharging degradation products.

Polypeptides to be degraded must wind their way through a system of internal cavities and constrictions until they reach the active sites in the central cavity, at least 8–10 nm away from the orifice at the center of the α ring (see Figure 3). The underlying mechanism of translocation is unknown, as is the precise role of the two antechambers. One could envisage that when the 20S proteasome is associated with regulatory complexes which unfold substrate proteins in an ATP-dependent manner, unfolding and translocation of the polypeptide are coupled, and thus, that the unfolded chain is "pushed" into the 20S core. However, when the unfolded polypeptide alone is offered to the 20S proteasome in vitro, it is capable of degrading it; thus, the translocation is not strictly energy-dependent. The structural properties of the interior of the proteasome should bias the random walk of the polypeptide chain

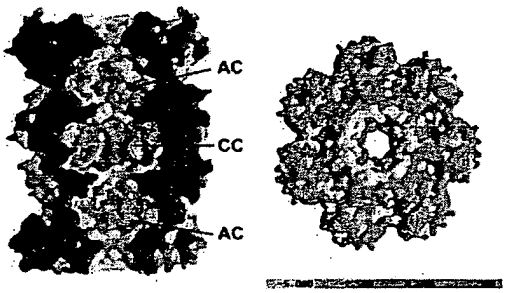


Figure 3. Hydrophobicity of Inner and Outer Surface Regions of the *Thermoplasma* 20S Proteasome

The hydrophobicity of the amino acids was taken from Pickett and Sternberg, 1993, and mapped to the molecular surface. The left side shows a cut-open model of the complex with the antechambers (AC) formed jointly by an α and β ring, and the central cavity (CC) formed by two β rings. The cutting surface is shown in dark gray. On the right side, the outer surface of one α ring is shown. The scale bar shows the color coding of the hydrophobicity in kcal/mol.

toward the active side clefts; the antechambers, which have a volume of ~59 nm³, must be able to maintain the polypeptide in an unfolded form as it passes through them. It is possible that the existence of constrictions segregating the front end from the rear end of a translocating polypeptide chain serves as a means to prevent refolding. The crystal structure of the chaperonin GroEL in complex with its co-chaperonin GroES (Xu et al., 1997; for review, see Bukau and Horwich, 1998, this issue of *Cell*) illustrates how properties of cavities may determine interactions with nonnative polypeptides. Upon binding of GroES, one of the two heptameric GroEL rings (the *cis* ring) undergoes a major structural rearrangement and, concomitantly, the properties of the *cis* cavity change: while the *trans* cavity is lined with hydrophobic residues, favoring the interaction with nonnative polypeptides, the *cis* cavity exposes mostly polar residues and thus repels nonnative polypeptides. In terms of overall hydrophobicity, the antechambers of the proteasome assume an intermediate position. Upon careful inspection, one may discern an array of paths where hydrophobic residues are clustered (Figure 3); in spite of their meandering appearance, these paths seem to connect the α-ring channel with the inner constriction and thus may give direction to a polypeptide chain on its way into the central cavity.

The central cavity, which is less hydrophobic than the antechambers, has a volume of ~84 nm³, allowing it, in principle, to accommodate a single folded protein of ~70 kDa; a loosely packed unfolded polypeptide requires much more space. Since polypeptides can only enter the cavity one after the other, the central cavity will not usually accommodate more than one polypeptide at a time. The confinement of the substrate to this cavity with its 6–14 active sites provides the structural basis for the processive mode of action of the proteasome; it completes the degradation of one polypeptide before attacking the next (Akopian et al., 1997).

Characteristics of the Degradation Products

Eukaryotic proteasomes have three major peptidase activities that have been defined using short fluorogenic peptide substrates: a chymotrypsin-like activity, which cleaves after hydrophobic residues, a trypsin-like activity, which cleaves after basic residues, and a peptidylglutamyl peptide-hydrolyzing activity, which cleaves after acidic residues. Two additional specificities have been identified in mammalian proteasomes, cleaving after branched chain residues and between small neutral amino acids (for review, see Cardozo, 1993). The proteasomes of *Thermoplasma* and of the bacterium *Rhodococcus* have only chymotrypsin-like activity, consistent with the fact that they have only one type of active site (Dahlmann et al., 1992; Tamura et al., 1995). Mutational studies and the crystal structure of the yeast proteasome can be used to assign these activities to distinct subunits (see Figure 2), but they have little (if any) relevance to the cleavage specificity of protein substrates. Degradation studies performed with *Thermoplasma* proteasomes (Wenzel et al., 1994) and eukaryotic proteasomes (e.g., see Dick et al., 1994; Ehring et al., 1996) have yielded cleavage patterns that do not correlate with the aforementioned specificities. Obviously, a classification of cleavage specificities using the residue directly adjacent to the cleavage site (the P1 position) falls short of reality.

It was intriguing to observe that, in spite of cleaving protein substrates in a rather nonspecific manner, the generated products fell into a relatively narrow size range, averaging around 7–9 residues. This property, which is common to prokaryotic and eukaryotic proteasomes, led to the proposal that an intrinsic "molecular ruler" determines product length (Wenzel and Baumeister, 1995). It was envisaged that the distance between active sites, acting in concert, could provide the physical basis of such a ruler. The crystal structure of the *Thermoplasma* proteasome revealed a distance of 2.8 nm between neighboring active sites corresponding to a hepta- or octa-peptide in an extended conformation; thus, it seemed to provide strong evidence in support of the molecular ruler hypothesis. On the other hand, recent more quantitative analyses of product lengths (Kisselev et al., 1998), while in agreement with the average length, showed larger size variations, which may be difficult to reconcile with a purely geometry-based ruler.

It is now well established that the proteasome has an important role in generating immunocompetent peptides to be displayed by the MHC class I complex. Obviously, the evolution of the proteasome predates the evolution of the immune system, and the availability of peptides between 7 and 9 residues long must have had a profound influence on the evolution of the MHC class I system (Niedermann et al., 1997). In turn, the proteasome seems to have responded to the need of the immune system for specific peptides by developing variants of some of its β-type subunits, which upon induction by γ-interferon can replace their constitutive counterparts in the 20S complex, thus allowing further modulation of specificity (for reviews, see Goldberg et al., 1995; Heemels and Ploegh, 1995).

Proteasomes Carry Cellular Localization Signals

Proteasomes are located both in the nucleus and in the cytoplasm of eukaryotic cells, and they undergo a cell cycle–dependent redistribution (Amsterdam et al., 1993; Palmer et al., 1994). Proteasomes indeed possess multiple nuclear localization signals (NLS sequences) that

are present on two (yeast) or four (human) α-type subunits. They are located on either one of two surface-exposed loops that are situated on the outer periphery of the α rings in the assembled complex. The functionality of these sequences has been demonstrated by means of reporter molecules to which peptides with the respective NLS sequences were coupled (Nederlof et al., 1995) as well as by mutational studies (Wang et al., 1997a). Signal-mediated changes in the cellular location of the proteasome add a layer of complexity to the spatial and temporal regulation of proteolysis.

The Propeptide Has Important Roles in the Assembly Pathway

The assembly of the 20S proteasome is intimately coupled to the processing of the β-type subunits, which renders them active. Posttranslational removal of a propeptide is a common mechanism for controlling the activity of proteolytic enzymes and perhaps best studied for extracellular bacterial proteases such as the α-lytic protease. Propeptides may have functions beyond preventing premature activation; they can promote folding, acting as intramolecular chaperones, or serve as signals that determine the cellular localization of the enzyme (for review, see Baker et al., 1993). In the proteasome, the proregion is removed at a late assembly stage, thus deferring activation until the β subunits are confined to the inner cavity of the complex. The cleavage reaction proceeds via an autocatalytic mechanism relying on the active site threonine (Thr-1). The invariant glycine preceding it (Gly-1) is the prime (if not the only) determinant of the cleavage site (Chen and Hochstrasser, 1996; Schmidtke et al., 1996; Seemüller et al., 1996). It is as yet unclear whether the autocatalytic reaction is intramolecular (*cis*) or intermolecular (*trans*) or whether both mechanisms apply. In the *Thermoplasma* proteasome, coexpression of mutant, inactive β subunits and wild-type β subunits leads to efficient processing of the inactive subunits, which demonstrates that intermolecular processing is possible (Seemüller et al., 1996). For eukaryotic proteasomes, where several subunits have propeptides much longer than the eight residues found in *Thermoplasma*, a two-step model has been proposed: in the first step, the propeptide is trimmed in size by an intermolecular mechanism; and in the second step, the residual propeptide of 8–10 residues is then removed via an intramolecular mechanism (Schmidtke et al., 1996).

Archaeal, bacterial, and eukaryotic proteasomes do not seem to follow exactly the same assembly pathways. This reflects the increase in complexity and the corresponding need in eukaryotes to orchestrate the orderly assembly of 14 different subunits. The sequence of events that lead from individual subunits to mature proteasomes are best understood for the *Thermoplasma* proteasome (Zwickl et al., 1994; Seemüller et al., 1996). Here, the α subunits assemble spontaneously into seven-membered rings in the absence of β subunits. In contrast, the β subunits alone are unable to assemble, remain unprocessed, and do not even fold completely. Thus, it appears that the α rings serve as a template upon which the β subunits assemble. Interestingly, at least one of the seven human α-type subunits forms ring structures resembling those of *Thermoplasma* α subunits (Gerards et al., 1997). Although it remains unproven that α rings are an assembly intermediate in vivo,

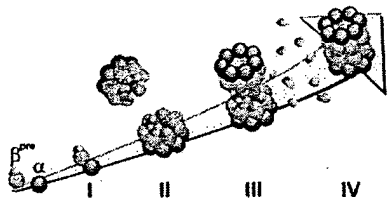


Figure 4. Assembly Pathway of the *Rhodococcus* Proteasome

The first step in the assembly pathway is the formation of α/β heterodimers (I) followed by the assembly of half proteasomes (II). Half proteasomes dimerize to form preholoproteasomes (III), which are converted into holoproteasomes (IV) through the autocatalytic removal of the propeptide.

a recent study analyzing precursor complexes of mouse proteasomes with subunit-specific antibodies lends support to such a role (Nandi et al., 1997). These authors propose that assembly is initiated by the formation of a ring containing all seven α-type subunits and then continues with the sequential addition of the seven β-type subunits. Recent experiments with the *Rhodococcus* proteasome suggest a variant pathway: while recombinant α- and β subunits assemble efficiently in vivo and in vitro, neither of the two components alone yields any detectable assembly product; hence, one must conclude that an α/β heterodimer is an early intermediate in the pathway (Zühl et al., 1997b).

Proteasome propeptides are not only highly variable in length and sequence, but also in their importance for the assembly process. While deletion of the 65 residue propeptide of *Rhodococcus* β subunits results in a drastically reduced yield of mature proteasomes (Zühl et al., 1997b), deletion of the 8 residue propeptide of the *Thermoplasma* β subunit has very little effect (Zwickl et al., 1994). Not surprisingly, therefore, *Thermoplasma* proteasomes can be reconstituted efficiently in vitro following complete dissociation or even unfolding (Grziwa et al., 1994). *Dictyostelium* proteasomes cannot be reconstituted in vitro, indicating that, once processed, the subunits have lost the ability to find their correct place in the complex (Schauer et al., 1993). In yeast, expression of the β (Doa3) subunit without its prosequence also results in a failure to incorporate this subunit into the complex (Chen and Hochstrasser, 1996). Since *Rhodococcus* proteasomes, which are composed of four subunits (α1, α2, β1, and β2), assemble very efficiently in vitro with any combination of α and β, this system offers a means to dissect the assembly pathway (Zühl et al., 1997b). Separately, *Rhodococcus* α and β subunits remain monomeric and inactive. When the subunits are allowed to interact, active proteasomes form spontaneously. Therefore, the earliest intermediate is probably an α/β heterodimer (I in Figure 4). The heterodimers assemble further into half proteasomes built from α subunits and β-subunit precursors (II in Figure 4). Previously described 13S–16S precursor complexes of mammalian proteasomes may represent the eukaryotic counterparts of these intermediates (e.g., see Yang et al., 1995; Schmidtke et al., 1997). Half proteasomes remain inactive even when the propeptide is absent due to genetic deletion. Assembly proceeds via dimerization of half

proteasomes and is completed by the final conversion of preholoproteasomes into holoproteasomes (III and IV in Figure 4). The last step involves the cleavage of the propeptide, which is the rate-limiting step in the assembly pathway. Deletion of the β-propeptide retards but does not completely prevent proteasome assembly; addition of the propeptide in *trans* can compensate for the deletion. In that case, formation of holoproteasomes from half proteasomes is accelerated, because the cleavage reaction becomes unnecessary. These observations indicate that the role of the propeptide is twofold: It supports initial folding of the β subunits, thus acting as an intramolecular chaperone, and it promotes the maturation of holoproteasomes following the docking of two half-proteasomes.

The Proteasome and Its Regulatory Complexes

The 19S Cap Complex Renders Proteolysis Energy-Dependent

In electron micrographs, the "26S" proteasome (a more accurate value is 30.3S; Yoshimura, et al. 1993) appears as an elongated structure (~45 nm long) consisting of a central 20S complex capped at either one or both ends by the 19S complexes. These 19S caps, which have a molecular mass of approximately 700 kDa, serve to recognize ubiquitylated proteins and to convert them into a form competent for degradation by the 20S core complex. When bound to the 20S complex, the two 19S caps face in opposite directions, reflecting the underlying C2 symmetry of the 20S proteasome (Peters et al., 1993). The coexistence of symmetric and asymmetric 26S complexes is reminiscent of the situation with GroEL–GroES chaperonin complexes, where there has been some controversy regarding the functional significance of the two species (Schmidt et al., 1994; Engel et al., 1995). Similar to the situation with GroEL–GroES, we do not see any compelling reason why a symmetric complex should be obligatory; in fact, an asymmetric complex seems to be better suited for performing a vectorial process involving the uptake of substrate and the release of product.

The 19S caps are flexibly linked to the 20S core complex. As illustrated in Figure 5a, they undergo a "wagging" type of movement; in symmetrical complexes, the movement of the two 19S caps appears to be noncorrelated (Walz et al., 1998). We are not yet in a position to say whether such a movement is functionally relevant and, perhaps, dependent on ATP-hydrolysis or whether it merely reflects some internal flexibility. It is noteworthy that very similar characteristics and amplitudes of movement are observed in 26S proteasome preparations isolated from different sources and in different types of EM preparations. In any case, the lability of the complex and the existence of such a flexible linkage make the structural analysis of the 26S proteasome a cumbersome task. A low-resolution 3D map has recently been obtained following segmentation of the complex on the level of image analysis; a composite model combining the separately determined structures of the 19S caps and the 20S core is shown in Figure 5b. The model provides a first insight into the remarkable complexity of the 19S structure. Although it may be of little immediate

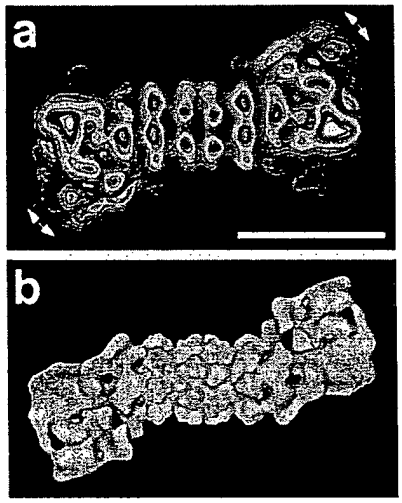


Figure 5. The 26S Proteasome

(a) Two-dimensional average of the 26S proteasome from *Drosophila melanogaster* obtained from electron microscopic images of negatively stained specimens. Image analysis of a large dataset of particles revealed the flexible linkage of the 19S caps to the 20S core. The figure shows two states of the complex at the maximum amplitude of the wagging movement superimposed, one in color-scale, the other in gray-scale. The direction of the motion is indicated by the white arrows. From statistical analysis, it can be deduced that the motions of the left and right 19S caps are not correlated. The scale bar is 20 nm.

(b) The 3D structure of negatively stained 19S caps was calculated using the method of random conical tilting. The figure shows a composite model of the electron microscopic structure of the 19S caps combined with the low-pass filtered 20S proteasome.

usefulness, it will serve as a platform for an accurate mapping of the approximately 20 different subunits and for monitoring, by time-resolved EM, the fate of substrates "en route" through this labyrinth of subunits.

Much progress has been made in recent years in defining the components of the 19S complex, which is similar if not identical to PA700 (a *p*roteasome *a*ctivator of 700 kDa [for review, see Peters, 1994]). Therefore, in compiling a listing of its component subunits (Table 1), we have not discriminated between the two complexes. Currently 15 different subunits (not including homologs) have been defined through sequencing; at least 3 more subunits have been identified by SDS-gel electrophoresis only. Six of the subunits are ATPases and members of the AAA-ATPase family (*A*TPases *a*ssociated with a variety of cellular *a*ctivities); the proteasomal ATPases form a distinct branch within this superfamily. A hallmark of the 19S cap ATPases, (with one possible exception, S4), is a predicted coiled coil segment near the N terminus (Lupas et al., 1993). Six genes encoding ATPases, which are members of this subfamily, are found in the yeast genome. In archaea, close relatives of the 19S cap ATPases have been discovered in the genomes of *Methanococcus jannaschii* and *Archeoglobus fulgidus*. In actinomycetes, a more distantly related ATPase of the AAA family has been found in the vicinity of the proteasome genes; when expressed in *E. coli*, it forms

Table 1. Subunits of the 19S Complex (Human and Yeast)

Subunit[a]	Name[b]	Accession[c]	Structural Properties/Sequence Similarities	Phenotypes of Mutants
S1 (110 kDa)[d]	p112 Sen3	D44466 L06321	contains KEKE motifs; similarity to S2 and subunits of the 20S cyclosome or anaphase-promoting complex/APC (Lupas and Baumeister, 1997)	mutation causes defects in ubiquitin-dependent proteolysis, cell-cycle progression, and nuclear protein transport; may interact with Nin1 (S14); Sen3 interacts with tRNA splicing factor Sen1 (DeMarini et al., 1995; Yokota et al., 1996)
S2 (100 kDa)	p97 (55.11, TRAP-2, p67) Nas1	D78151 U10399	contains KEKE motifs; similarity to S1 and subunits of the 20S cyclosome or anaphase-promoting complex/APC (Lupas and Baumeister, 1997)	55.11 (TRAP-2) binds to TNF receptor type1 and may be involved in induced cell death (Boldin et al., 1995); Nas1 mutants accumulate ubiquitylated proteins (Tsurumi et al., 1996)
S3 (61 kDa)	p58 Sun2	DDBJ:67025 U18778	—	Sun2 may function in cell cycle similar to Nin1 (S14); mutants accumulate ubiquitylated proteins (Kominami et al., 1997)
S4 (52 kDa)	p56 Yta5	L02426 X81070	member of AAA ATPase superfamily (Confalonieri and Duguet, 1995); binds to S7 in vitro (Richmond et al., 1997)	—
S5a (50 kDa)	S5a (AF-1) Mcb1 (Sun1)	U51007 D78022	contains KEKE motifs; similarity to human BTF2/p44 and homologs (basic transcription factors)	although this subunit shows in vitro high affinity to polyubiquitin (Deveraux et al., 1997; Kominami et al., 1997), it seems not to be essential for ubiquitin-dependent proteolysis (van Nocker et al., 1996); S5a restores DNA binding capacity of HLH proteins MyoD and E12 by interacting with Id1 (Anand et al., 1997)
S5b (50 kDa)	S5b no yeast homolog	S79862	contains 9 dileucine repeats	—
S6 (48 kDa)	p48 (Tbp7) Yta2	Sp: P43686 U06229	member of AAA ATPase superfamily; binds to S8 in vitro (Richmond et al., 1997)	Tbp7 was identified as HIV-Tat binding protein (Dubiel et al., 1994)
S6' (48 kDa)	p50 (Tbp1) Yta1	M34079 Z78025	member of AAA ATPase superfamily; binds to S10b in vitro (Richmond et al., 1997)	Tbp1 (HIV-Tat binding protein) is a putative transcriptional activator (Ohana et al., 1993)
S7 (47 kDa)	Mss1 Cim5 (Yta3)	D11094 Z22817	member of AAA ATPase superfamily; binds to S4 in vitro (Richmond et al., 1997)	overexpressed Mss1 enhances Tat-mediated transactivation of HIV genes (Shibuya et al., 1992); cim5 mutation stops cell division and causes accumulation of B-type cyclins (Ghislain et al., 1993)
S8 (46 kDa)	p45 (Trip1) Sug1 (Cim3)	L38810 X66400	member of AAA ATPase superfamily; binds to S6 in vitro (Richmond et al., 1997)	Sug1 is a putative transcriptional mediator (Lee et al., 1995; Xu et al., 1995; Rubin et al., 1996; Wang et al., 1996; Fraser et al., 1997; Weeda et al., 1997); Cim3 mutation stops cell division and causes accumulation of B-type cyclins (Ghislain et al., 1993)
S9 (46 kDa)	S9 Orf	AF001212 X95644	contains 9 dileucine repeats	—
S10a (44 kDa)	S10a (Orf07) Orf	D14663 U32445	similarity to *Arabidopsis* fus6 and homologs in human and rat (Fusca proteins)	Fusca proteins are critical for plant development (Castle and Meinke, 1994)
S10b (44 kDa)	p42 Sug2 (Pcs1)	D78275 U93262	member of AAA ATPase superfamily; binds to S6' in vitro (Richmond et al., 1997)	Sug2 was identified as suppressor of *gal4* (Russell et al. 1996); mutants fail to duplicate the spindle pole body (McDonald and Byers, 1997)
S11 (43 kDa)	unknown	—	—	—
S12 (36 kDa)	p40 (S12) Nas3 (YOR261c)	D50063 Z75169	contains KEKE motifs	—
S13 (32 kDa)	unknown	—	—	—
S14 (30 kDa)	p31 Nin1	D38047 D10515	—	Nin1 is required for activation of cdc28 kinase; mutation stops cell cycle (Gordon et al., 1996; Kominami et al., 1997)
S15 (25 kDa)	unknown	—	—	—

[a] Subunits are numbered according to their SDS-PAGE mobility (Dubiel et al., 1995).
[b] First line: name of the human subunit; second line: name of the yeast subunit.
[c] Genbank accession; exceptions: DDBJ, DNA database of Japan; Sp, Swiss-protein.
[d] SDS-PAGE apparent molecular mass, ± 3 kDa depending upon the gel system (Dubiel et al., 1995).

a ring-shaped complex with ATPase activity (Wolf et al., 1998). This suggests that the proteasomal ATPases form six-membered homomeric rings in archaea and bacteria, and six-membered heteromeric rings in eukarya. From the structure of simpler ATP-dependent proteases, such as HslVU and ClpAP, it has been inferred that in the 19S cap complex, they also form a ring docking directly onto the α rings and surrounding the orifice of the 20S channel. Although plausible, rigorous experimental proof for such a topology is currently lacking. Also, the precise role of the ATPases, that is, the energy-dependent step in the degradation of proteins, awaits

clarification. It may include the recognition of target proteins, their dissociation and unfolding, their translocation into the 20S inner cavities, and/or the gating of the channel. Dissociation and unfolding are particularly likely steps of ATP involvement; however, an unfoldase activity has yet to be demonstrated.

19S cap complexes have only been found in eukaryotic cells, indicating that the non-ATPase subunits of the complex have been added to the proteasome-ATPase complexes late in evolution. Several of them are required to link the proteasome to the ubiquitin system (ubiquitin-binding, deubiquitylation), which through its array of ligating enzymes (for review, see Hochstrasser, 1996) confers specificity to the proteasome. This, however, does not imply that the proteasome acts exclusively in the context of the ubiquitin pathway. The functions of most of the non-ATPase subunits of the 19S cap complex are still unknown (Table 1). The large variety of phenotypes associated with disruption of their genes is a reflection of the involvement of the proteasome in a large number of cellular processes but gives only vague hints as to their biochemical function.

The 11S Complex: An ATP-Independent Activator

The regulator, variously referred to as 11S or PA28, was originally identified as an activator of the peptidase activities of the 20S proteasome from mammalian cells (Chu-Ping et al., 1992; Dubiel et al., 1992). It has no hydrolytic activity of its own, but when combined with 20S proteasomes it accelerates the degradation of fluorogenic peptides in an ATP-independent manner. However, it does not stimulate the degradation of proteins or of ubiquitin-protein conjugates. Since free peptides are short-lived and therefore rare in cells, the in vivo relevance of this complex is not obvious. Furthermore, its occurrence seems to be restricted to higher eukaryotes—no homolog exists in yeast—indicating that it has a specific rather than a basic function in the proteasomal degradation pathway. Most likely, these functions are related to the proteasome's involvement in antigen processing. The first evidence for a role in this context came from experiments demonstrating that the synthesis of 11S/PA28 is strongly induced by the cytokine γ-interferon (Realini et al., 1994). Similarly, γ-interferon induces synthesis of three β-type subunits of the 20S proteasome, LMP2, LMP7, and MECL-1, which replace their constitutive counterparts (see Figure 2) in the "immunoproteasome." Meanwhile, further evidence has accumulated supporting a role of 11S/PA28 in antigen processing. The cleavage of fluorogenic peptides by LMP2/7 containing proteasomes is enhanced in its presence (Ustrell et al., 1995), and the repertoire of peptides generated from a polypeptide becomes larger (Groettrup et al., 1995). Using natural peptides, it was shown that the presence of PA28 changes the single-cleavage mode of 20S proteasomes to a coordinated double-cleavage mechanism, thereby optimizing the generation of dominant T-cell epitopes (Dick et al., 1996b). Finally, the observation that overexpression of PA28 to a level similar to that obtained after γ-interferon treatment markedly enhanced the efficiency of viral antigen processing establishes the role of PA28 in vivo (Groettrup et al., 1996). Recently it was proposed, based on experiments with the HIV-1 Tat protein, that viruses may escape from immune surveillance by preventing the formation of 20S/11S complexes (Seeger et al., 1997). Although the role of PA28 in antigen processing appears to now be well established, the mechanism by which PA28 modulates the activity of the 20S proteasomes remains enigmatic.

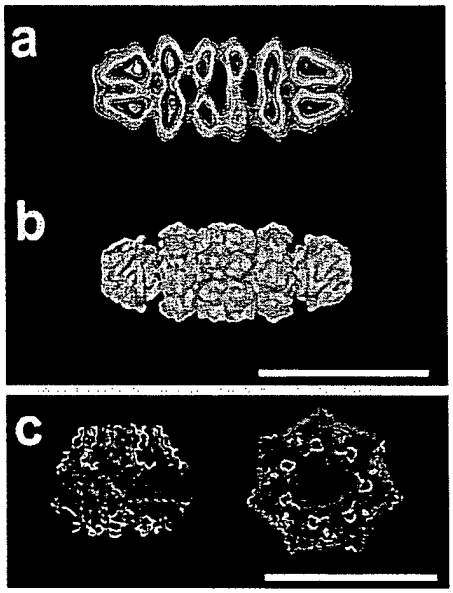


Figure 6. The 11S/PA28 Regulator

(a) Two-dimensional average of the PA28–20S complex from the rat.

(b) Composite surface model of the PA28 regulatory complex derived from the atomic coordinates and the 20S proteasome. The scale bar is 20 nm.

(c) Ribbon model of the atomic structure of the recombinant human PA28 complex as determined by X-ray crystallography (Knowlton et al., 1997). Side view (left) and top view (right). The scale bar is 10 nm.

Structurally, PA28 forms multimeric ring-shaped complexes (Gray et al., 1994) that bind to the two α rings of the 20S proteasome (Figure 6a). It is built from two types of subunits of approximately 30 kDa, PAα and PAβ, which are about 50% identical in sequence. Biochemical data suggest that PAα and PAβ form heteromeric rings containing the two subunits in a 1:1 stoichiometry (Ahn et al., 1996; Kuehn and Dahlmann, 1996; Song et al., 1996); this would argue for an even number of subunits, most likely six, in the complex. Recombinant PAα assembles into seven-membered rings (Knowlton et al., 1997), which are able to stimulate 20S proteasomes similarly to the native PAα/β (Realini et al., 1994; Song et al., 1996). PAβ alone does not form multimers nor does it exhibit stimulatory activity (Kuehn and Dahlmann, 1996). This indicates that PAα mediates association within the 11S complex and between this complex and the 20S proteasome; a short carboxyl-terminal sequence of PAα was found to be critical for this role (Song et al., 1996). Interestingly, the two PA28 subunits have sequence similarity (about 35% identity) to a third protein, the nuclear Ki antigen found in systemic lupus erythematosus patients. The function of this protein,

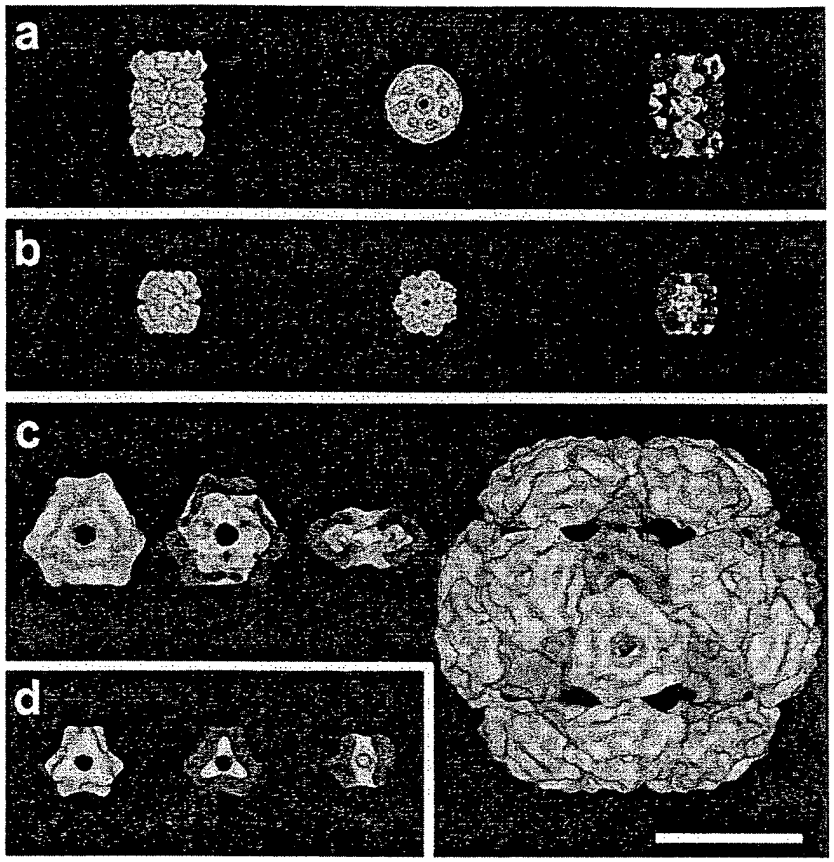


Figure 7. Synopsis of Structures of Self-Compartmentalizing Proteases Represented as Surface Models with Equal Scaling

(a) 20S proteasome of *Thermoplasma acidophilum* (Löwe et al., 1995). Side view (left), top view (center), and cut-open side view with the proteolytic sites marked in red (right).

(b) ClpP protease from *E. coli* (Wang et al., 1997b).

(c) Tricorn protease from *Thermoplasma acidophilum* (Walz et al., 1997). On the left, a single hexameric tricorn molecule is shown; it was taken from the 3D reconstruction of the icosahedral tricorn capsid (right).

(d) Gal6/bleomycin hydrolase from yeast (Joshua-Tor et al., 1995).

The structures in (a) and (b) were low-pass filtered to 1.2 nm, and the structures in (c) and (d) to 2.5 nm. The scale bar is 20 nm.

which is highly conserved among species, is still unknown. Its relationship to the subunits of PA28 and the fact that it is upregulated by γ-interferon, points to a possible role in immunity. However, unlike PAα and β, the occurrence of the Ki antigen is not restricted to higher vertebrates (Paesen and Nuttall, 1996).

Recently, the crystal structure of a heptameric complex of PA28α has been solved (Figure 6c). The PA28α monomer is almost exclusively α-helical; three of its four long helices form a tightly packed cone, the fourth helix (H1) contains a sharp kink allowing it to interconnect to the neighboring monomer. The complex is traversed by a channel of 2 nm in diameter at its apex and 3 nm at the base, which docks to the proteasome (Figure 6b). It is suggested that the binding of PA28 causes a conformational change that opens the α-ring channel of the proteasome (Knowlton et al., 1997). However, this alone can hardly explain all the aforementioned modulatory effects of this regulator. In fact, it may have more far-reaching allosteric effects, perhaps even transmitted to the active sites in the central cavity. We observed that the binding of PA28 weakens the interaction between rings in the 20S proteasome to the extent of partial disassembly (W. B., unpublished data); this may facilitate the efflux of degradation products and, as part of a functional cycle, may prepare the proteasome for another round of degradation (Lupas et al., 1995).

Other Self-Compartmentalizing Proteases

Self compartmentalization has begun to emerge as a principle common to several proteases. The recently determined crystal structure of ClpP (Wang et al., 1997b) revealed a striking example of evolutionary convergence. In spite of being unrelated in sequence and fold, it forms a homooligomer built of two heptameric rings. This encloses a cavity with dimensions very close to

those of the central cavity of the 20S proteasome, which is accessible through an axial channel approximately 1.2 nm in diameter (Figure 7). Even the disposition of active sites, here formed by a classical Ser-His-Asp catalytic triad, and the distance between them (2.6 nm) almost matches the geometry inside the proteasome. Also, Clp is synthesized as a precursor, which is processed autocatalytically to its mature form. Like the 20S proteasome, ClpP associates with an ATPase, ClpA, which docks to both ends where the entry–exit channel is located (Kessel et al., 1995).

Recently, two energy-independent large protease complexes have been described, which are traversed by a channel that widens into a large central cavity: bleomycin hydrolase/Gal6 (Joshua-Tor et al., 1995) and the tricorn protease (Tamura et al., 1996). Bleomycin hydrolase is a papain-type cysteine protease; the active site of the tricorn protease is not yet fully defined. The 720 kDa tricorn hexamer, which has trypsin-like peptidase activity, assembles further in vivo to form an icosahedral capsid composed of 20 hexamers. This 15 MDa complex is likely to act as an organizing center, or scaffold, for several aminopeptidases. Interaction with the tricorn core complex enhances, possibly by an allosteric mechanism, intrinsic activities of these peptidases and elicits new ones found neither in the aminopeptidases nor in the tricorn alone.

Conclusions and Outlook

In recent years, it has become clear that the proteasome is a highly conserved proteolytic complex common to all three domains of life. In eukaryotic cells, the 20S proteasome is the core complex of an energy-dependent protein degradation machinery that equals the protein synthesis machinery in its complexity. While the ubiquitin-proteasome pathway of protein breakdown is essential for eukaryotes, prokaryotes possess redundant proteolytic systems. The principle of self-compartmentalization is common to many of them. In view of this redundancy, it is not surprising that proteasomes are not essential in archaea and bacteria under normal (i.e., nonstress) growth conditions. At present, the biology of proteolysis is not well understood for prokaryotes; it is largely unknown what the natural substrates are, how these substrates are targeted, and to what extent the substrate spectra of the various energy-dependent proteolytic systems might overlap.

Prokaryotic proteasomes have, by virtue of their relative simplicity, taken a pivotal role in elucidating the structure and enzymatic mechanism of the 20S complex. In spite of these advances, several important issues remain unresolved. We do not yet know how, precisely, the polypeptide chain is translocated into the barrel-shaped complex, nor do we know how the degradation products are discharged. Is some kind of ratcheting mechanism involved in directing the substrates to the proteolytic nanocompartment? If so, how does it work in molecular terms? Another major challenge is to achieve a better understanding of the principles that govern the ordered assembly of the 14 distinct but very similar subunits. The finding in higher eukaryotes that inducible subunits can replace constitutive subunits further adds to the complexity of the assembly pathway.

In eukaryotic cells, the physiologically relevant form of the proteasome is the 26S complex. The "parts list" of the regulatory 19S complex may be near completion now, but the precise function of the vast majority of these parts remains enigmatic, and hitherto no blueprint exists describing how the parts are assembled. It poses a considerable challenge to structural biologists to establish the structure and topology of the 19S subunits, particularly in view of the flexibility and variability revealed by initial studies. The ultimate goal must be to achieve a precise description of the sequence of events from the recognition of a ubiquitylated substrate protein to its translocation into the 20S proteolytic core. Like the ribosome and the spliceosome, the 26S proteasome is known to be an energy-dependent machinery. However, the popular but unproven hypothesis that ATP hydrolysis is used to fuel the disassembly and unfolding of substrate proteins awaits experimental verification.

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